N-methyl-Δ'-pyrrolinium salt (Fig. __.4). Dewey et al. (1955) and Leete (1955) fed 2-^{14}C-ornithine to tobacco and found nicotine to be symmetrically labeled at C-2' and C-5'. Symmetrical incorporation of 2-^{14}C-ornithine could occur from a free symmetrical intermediate such as putrescine. S-adenosylmethionine was an effective *in vivo* methyl donor for putrescine N-methyltransferase measurements (Mizusaki et al., 1971) and is most likely the in vivo methyl donor.

Yoshida and Mitake (1966) and Yoshida (1969a; 1969b) demonstrated that arginine, agmatine and N-carbamylputrescine, in sequence, were efficiently incorporated into putrescine. It has been generally accepted that this pathway was important in higher plants with the exception of the Solanaceae where decarboxylation of ornithine contributed significantly to putrescine formation (Leete, 1980). Berlin (1981) has shown in tobacco cell cultures that arginine and ornithine were equally efficient precursors of putrescine for cinnamoyl putrescine formation. Arginine decarboxylase and ornithine decarboxylase activities were nearly the same in cell cultures, and by use of inhibitors and substrates he concluded that putrescine was formed by initial decarboxylation of each substrate and not interconversion.

Yang (1984) isolated ornithine and arginine decarboxylase from root tissue of intact plants and found about 4-times as much activity of ornithine as arginine decarboxylase activity. Removal of the apical meristem (induction of nicotine accumulation in the plant) increased ornithine and arginine decarboxylase activity but after three days the activity of arginine decarboxylase was the same in intact and induced plants, whereas activity of ornithine decarboxylase remained higher for at least 23 days.

The incorporation of nicotinic acid into nicotine is better understood. Dawson et al. (1960a, 1960b) showed that nicotinic acid was incorporated into the pyridine ring of nicotine with loss of the carboxyl group (Fig. __.5). Also, Yang et al. (1965) showed that the pyrrolidine ring of nicotine was attached at the C-3 position of the pyridine ring, the position from which the carboxyl group was lost. Dawson et al. (1960a) also showed that the hydrogen at position C-6 of nicotinic acid was lost during nicotine formation. From the above observations Dawson and Osdene (1972)

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and Leete (1977) presented the hypothesis for nicotine formation that included the reduction of nicotinic acid to 3,6-dihydronicotinic acid.

Friesen and Leete (1990) proposed that the dihydronicotinic acid yielded a zwitterion (a β-iminium carboxylic acid) by proton transfer which readily decarboxylates to yield 1,2-dihydropyridine. 1,2-Dihydronicotine reacts with N-methyl-Δ'-pyrrolinium salt to yield 3,6-dihydronicotine. The 3,6-dihydronicotine is oxidized with loss of the hydrogen originally present at C-6 of nicotinic acid and retention of the hydrogen added in the reduction step to 3,6-dihyronicotinic acid (Fig. __.5). All nicotine in tobacco plants is the (-)-2'S-nicotine.

A minor biosynthetic pathway for nicotine from nornicotine has been described by Leete (1984). He fed (RS)-[2'-^{14}C] nornicotine to *N. tabacum* and recovered small amounts (0.045%) of the radioactivity in nicotine with all ^{14}C still at the 2' position. The data indicate direct methylation of nornicotine to nicotine and Leete suggested putrescine methyltransferase may be able to catalyze this methylation though no data were presented to support this hypothesis.

Anatabine. Both pyridine rings of anatabine are formed ultimately from the two methylene carbons and nitrogen of aspartate and two carbons from a glycerol derivative via nicotinic acid as outlined for nicotine (Leete 1975, 1977, 1978; Leete and Slattery, 1976) (Fig. __.6). Incorporation of labeled precursors suggest the reduction of nicotinic acid to 3,6-dihydronicotinic acid and decarboxylation to form 1,2-dihydropyridine and 2,5-dihydropyridine. 1,2-Dihydropyridine reacts with the electrophile 2,5-dihydropyridine to yield 3,6-dihydroanatabine which is aromatized to (-)-2'S-anatabine (Leete and Muller, 1982). In this proposed biosynthesis the point of attachment between the two pyridine rings is the C-3, or the carbon from which decarboxylation occurred, to C-2 or C-6 of 2,5-dihydropyridine formed by the decarboxylation step. Even though anatabine and anabasine differ by only two hydrogens, there does not seem to be any interconversion between these two alkaloids.

Anabasine. The pyridine ring of (-)-2'S-anabasine has been shown to originate from nicotinic acid with loss of the carboxyl group as proposed for the pyridine ring of nicotine (Solt et al., 1960). The most accepted precursor for the piperidine ring is

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lysine via Δ'-piperidine with the nitrogen derived from the ε-nitrogen of lysine (Leete et al., 1964). Incorporation studies with 2-^{14}C-lysine have shown that the ^{14}C was incorporated into the C-2' position of the piperidine ring of anabasine (Leete, 1958, 1980). This result is consistent with a bound cadaverine throughout steps with a potential symmetrical molecule, and is contrasted to the apparent free putrescine in N-methylpyrrolidine ring formation for nicotine. Leete (1977) proposed the same mechanism for anabasine formation from nicotinic acid and Δ'-piperidine as for nicotine formation from nicotinic acid and N-methyl-Δ'-pyrrolinium salt.

Nornicotine. Nornicotine seems to be formed irreversibly from nicotine in green leaves or during senescence (Dawson, 1951). Demethylation of (-)2'S-nicotine in tobacco yields (-)-2'S-nornicotine and (+)-2'R-nornicotine in nearly equal amounts. Data of Leete and Chedekel (1974) support a demethylation mechanism involving an iminium salt and tautomeric shift to obtain racemization of nornicotine. Their results demonstrate that the H atom at C2' of (-)-2'S-nicotine is retained in formation of (-)-2'S-nornicotine, but is lost in formation of (+)-2'R-nornicotine (Fig. __.5). They also showed that the naturally occurring (-)-2'S-nicotine and (+)-2'R-nicotine were equally effective as substrate for the in vivo demethylation enzyme. Kisaki and Tamaki (1961) suggested that the amount of racemization of nornicotine is variable and may be influenced by environmental conditions and maturity or senescence of the plant. N-demethylation of nicotine is a significant step in alkaloid metabolism in several *Nicotiana spp.* as it is the last step in production of nornicotine. Demethylation of nicotine to nornicotine has been described to be controlled by one of two genes. Characterization of the enzyme would provide potential plant modification for formation and accumulation of N'-nitrosonornicotine (NNN) and N'-acylnornicotines in tobacco products. The N-demethylation of nicotine is coupled with the oxidation of the methyl group to CO_2 (Stepka and Dewey, 1961) and was reported to be the primary source of nornicotine by Dawson (1945). A preliminary report by Chelvarajan et al. (1991) indicates that nicotine demethylase activity is associated with the microsomal fraction of the cell. Demethylation was dependent on the presence of reduced nucleotides. The activity of nicotine demethylase was over 3-times higher

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when NADPH was substituted for NADH. In vitro pH and temperature optima were 7.0 to 7.5 and 30°C, respectively. Higher concentrations of nornicotine in the assay decreased the rate of nicotine demethylation. Nornicotine was a more effective inhibitor of demethylation than an identical concentration of nicotine suggesting product inhibition rather than a general alkaloid effect. Vmax and the apparent Km for nicotine were estimated to be 11 pmoles min^{-1} and 118 μM, respectively. Reports indicate that many N-demethylases in higher plants are associated with cytochrome P-450. Addition of tetcyclasis, a cytochrome P-450 type enzyme inhibitor (Canivenc et al., 1989), resulted in partial inhibition of demethylation of nicotine. Carbon monoxide also inhibits many P-450 enzymes and the inhibition can often be reversed by light of 450 nm (Donaldson and Luster, 1991), but in this study CO did not inhibit nicotine demethylation. However, an antibody to Jerusalem artichoke NADPH cytochrome P-450 reductase inhibited *in vitro* nicotine demethylation suggesting that the enzyme may be a P-450 type enzyme (Benveniste et al., 1986). These data are equivocal and further characterization is required.

In the growing plant, nornicotine may be dehydrogenated to myosmine (Kisaki and Tamaki, 1966; Leete and Chedekel, 1972). Leete (1984) fed ^{14}C-nornicotine to intact plants for 8 days and found 55% of the radioactivity in nornicotine and 15% in myosmine. After feeding ^{14}C-2'-myosmine to *N. glauca*, no labeled nicotine or nornicotine was found, suggesting that the dehydrogenation of nornicotine to myosmine was irreversible. However, activity was found in nicotinic acid and with most of the label in the carboxyl carbon (Fig. __.5).

Regulation of Nicotine Content in Tobacco Plants

Genetic. Genetic control of alkaloid levels in tobacco has received much attention and will continue to be an area of intensive research. Identification of the gene systems responsible for tobacco alkaloid accumulation expands the possibility of success of genetic engineering programs to control alkaloid production in plants or cell culture. Two principal genetic systems for regulating the level and kinds of alkaloids in tobaccos have been described.

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The level of total alkaloids (nicotine) in tobacco is determined by genes at two loci (Legg et al., 1969; Legg and Collins, 1971). These two loci are designated A and B. Commercial cultivars are considered homozygous dominant at the two loci (AABB) and low alkaloid genotypes homozygous recessive (aabb) at the two loci. Legg and Collins (1971) obtained the nine possible genotypes for the two locus model and demonstrated that the relative dosage effect of the homozygotes at locus A were 2.4 times greater than the homozygotes at locus B. Dominant alleles at both the A and B alkaloid loci were associated with higher levels of total alkaloids, total-N and total volatile nitrogenous bases, but lower levels of protein-N, than were the recessive alleles. The decreased total-N content associated with the recessive alleles compared to the dominant alleles was approximately equal to the decreased alkaloid-N, and the differences in total volatile nitrogenous bases were mainly due to differences in the alkaloid-N fraction. Significant genotypic differences were detected for several plant characteristics but no consistent ranking of genotypes was evident.

The other genetic system controls the conversion of nicotine to nornicotine in the plant. Nornicotine is produced by demethylation of nicotine (Dawson, 1952). There are two potential loci for nicotine demethylation in commercial tobacco, *N. tabacum*. One locus is found in the progenitor *N. tomentosiformis* genome and the other in the progenitor *N. sylvestris* genome (Mann et al., 1964). The primary site of nicotine demethylation is the leaf; however, the demethylation gene from *N. tomentosiformis* causes nicotine to be converted to nornornicotine prior to senescence while the gene from *N. sylvestris* caused the conversion to occur during senescence (Wernsman and Matzinger, 1968). Plants with $c_1c_1c_2c_2$ genotypes are not capable of demethylating nicotine to nornicotine. Only one dominant allele at either locus is necessary for demethylation to occur. The C_1 locus from *N. tomentosiformis* appears to be active in the nicotine to nornicotine conversion in commercial cultivars but the high mutation rate at this locus is for nornicotine formation during senescence and not in the green plant as in the original *N. tomentosiformis* gene (Griffith et al., 1955; Burk and Jeffrey, 1958; Mann et al., 1964).

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Physiologic. In the recent past the biochemist and physiologist have used these proposed pathways to propose enzyme systems and to provide mechanisms by which the biosynthesis actually occurs in the plant. To date, most of this research has been focused on understanding nicotine biosynthesis and the regulation of nicotine biosynthesis in the plant.

Mizusaki et al. (1973) reported activities of three enzymes in the proposed sequence to 4-methylaminobutanal formation (Fig. __.4). They found ornithine decarboxylase (ODC), putrescine N-methyltransferase (PMT), and N-methylputrescine oxidase (MPO) activities to be high in tobacco roots (primary site of alkaloid biosynthesis), but low or no detectable activities in tobacco leaves. Root enzyme activities increased 2 to 16-fold in 24 hr following nicotine induction (plant decapitation). Saunders and Bush (1979) reported PMT and MPO increased after induction of high alkaloid genotype plants but MPO did not increase after induction of low alkaloid genotype plants. Pudliner (1980) used citrate synthase as a check for general plant metabolic activity and found ODC and citrate synthase activities increased after induction of both high and low alkaloid genotypes, but there were no differences between the two genotypes.

PMT activity was proportional to leaf nicotine content in related and unrelated high and low nicotine accumulation genotypes and tissues (Yoshida, 1973; Saunders and Bush, 1979; Wagner et al., 1986b). Activities of ODC and MPO were not proportional to nicotine content. Data from Saunders and Bush (1979) and Feth et al. (1986) strongly suggest PMT is the enzyme under stringent control for the pyrrolidine moiety of nicotine biosynthesis.

The role of putrescine supply and utilization may be a regulatory step in biosynthesis of the pyrrolidine moiety. A major branch point on the biosynthetic route to nicotine appears to exist at putrescine, where at least four pathways utilizing this diamine diverge (Fig. __.7). Two important alternate events seem to be the methylation (Mizusaki et al., 1971) and N-aminopropylation (Baxter and Coscia, 1973) of putrescine as steps in the biosynthesis of nicotine and polyamines, respectively. The cinnanoyl putrescines constitute as much as 6% of the dry weight of

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cell cultures (Schiel et al., 1984) and levels of γ-aminobutyrate may be as much as 80 mg per plant (Tso and McMurtrey, 1960).

Putrescine levels are normally low in tobacco, but elevated levels have been reported to be associated with several mineral deficiencies (Yoshida, 1967). ODC activity was 10-times higher in tobacco callus than in roots of nicotine induced tobacco plants (Pudliner, 1980). High ODC activity and high polyamine levels are associated with rapid cell proliferation of tobacco callus but not high nicotine accumulation.

Tobacco callus contains low alkaloid levels compared to plant tissue. High ODC activity in callus suggests that putrescine formation is not limiting nicotine biosynthesis, but perhaps the utilization of putrescine and S-adenosylmethionine for polyamine biosynthesis, is limiting availability of putrescine for nicotine biosynthesis. Mizusaki et al. (1973) did not detect PMT in callus. Since this enzyme specifically catalyzes the formation of N-methylputrescine from putrescine and S-adenosylmethionine, it suggests that nicotine biosynthesis in callus is limited by methylation of putrescine.

Yang (1984) showed ODC activity in roots increased 8-fold within 24 hr of plant induction and then after 3 days decreased to a level 3-times that in noninduced plants and remained at this level above the control plants for at least 23 days. Increased ODC activity and the subsequent putrescine formation could be a contributing factor in the observed increased nicotine accumulation following induction.

Madsen et al. (1984) reported twice the amount of polyamines in senesced leaves of a low alkaloid genotype than in leaves of the isogenic high alkaloid genotype. Work of Ravishankar and Mehta (1982) support the premise that nicotine production is dependent upon putrescine and ornithine availability. They found urea enhanced nicotine production and ornithine aminotransferase activity in callus while ornithine transcarbamylase activity was suppressed. This suggests more availability of ornithine for nicotine biosynthesis. The inhibition of ornithine transcarbamylase may be due to putrescine formation via ornithine decarboxylase (Stalon et al., 1972).

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Examination of the substrate specificity of partially purified MPO of *N. tabacum* revealed that putrescine and cadaverine were both utilized 40% as readily by the enzyme as N-methylputrescine (Mizusaki et al., 1972). Since oxidation of putrescine to 4-aminobutanal could lead to formation of nornicotine, some explanation - be it compartmentalization, differential degradation rates of the two alkaloids, or removal of the 4-aminobutanal by oxidation to 4-aminobutyric acid - is needed to account for the disproportionate differences in nicotine and nornicotine content in nonconverter tobacco genotypes. Subsequently, root protein fractions from nicotine and nornicotine accumulator genotypes of *N. tabacum* were found to oxidize putrescine and N-methylputrescine to the same relative extent, suggesting that a difference in substrate specificity is probably not a major factor in different alkaloid accumulation patterns (Saunders and Bush, 1979). Regulation of nicotine or nornicotine biosynthesis by MPO seems unlikely from available data, although Feth et al. (1986) suggest MPO is regulated in a concerted manner with PMT.

Another potential physiologic regulation of nicotine biosynthesis could be mediated through nicotinic acid availability. For biosynthesis of the pyridine ring, quinolinic acid (pyridine-2,3-dicarboxylic acid) is an efficient precursor (Yang et al., 1965). High levels of quinolinic acid phosphoribosyltransferase (QPT) in roots but not leaves of intact nearly mature *N. rustica* plants suggested that this enzyme was utilized in nicotine biosynthesis (Mann and Byerrum, 1974). In contrast, levels of nicotinic acid phosphoribosyltransferase and nicotinamide deaminase in the same tissue were within the range of activity seen in a few species that do not accumulate pyridine alkaloids. QPT activities in root tissue from four *N. tabacum* genotypes having different alkaloid accumulations were proportional to leaf nicotine levels (Saunders and Bush, 1979). Enzyme activity increased within 24 hr after induction for all genotypes except the homozygous recessive low alkaloid genotype. Pudliner (1980) and Wagner et al. (1986a, 1986b) reported that QPT activity was greater in induced nicotine accumulating tissues and in genotypes with higher nicotine content than in non-induced tissues or low nicotine genotypes. The differential increase in QPT activity in these experiments was uniquely different as citrate synthase increased in both induced

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high and low alkaloid genotypes, but there was no difference between genotypes for citrate synthase activity (Pudliner, 1980).

The general conclusion from these data is that QPT is the main regulatory enzyme for nicotinic acid production in nicotine biosynthesis. In addition, Wagner et al. (1986a) demonstrated that the nicotinic acid pool increased (1) by synthesis of nicotinanide adenine dinucleotide (NAD) and degradation via nicotinamide mononucleotide and (2) by a more direct route from nicotinic acid mononucleotide by a glycohydrolase (Fig. __.8). The Km for the nicotinic acid mononucleotide glycohydrolase is high (~ 4mmol L^{-1}) and probably prevents complete depletion of the nicotinamide mononucleotide pool to ensure continued NAD availability under conditions of rapid nicotine accumulation. We have not found that nicotinic acid mononucleotide glycohydrolase has any apparent regulatory function in nicotine or anabasine biosynthesis and accumulation in *N. tabacum* and *N. glauca* (Fannin and Bush, 1988). Wagner et al. (1986b) also concluded that NAD-pyrophosphatase, nicotinamide mononucleotide glycohydrolase and nicotinic acid phosphoribosyltransferase have a secondary regulatory function in nicotinic acid formation.

In commercial (AABB genotype) tobaccos, nicotine synthase may be rate limiting since addition of methylputrescine and nicotinic acid do not significantly increase the accumulation of nicotine as they do in low alkaloid tobaccos. Friesen and Leete (1990) detected nicotine synthase activity in a cell-free system; however, their report contained minimal in vitro characterization of the enzyme. The enzyme was absent during the first 100 hr of seed germination. This absence of activity is consistent with the observations of nicotine changes in germinating tobacco seeds (Weeks and Bush, 1974). Friesen and Leete (1990) found nicotine synthase to be active at a narrow range near pH 7.0, stimulated by Mg^{2+}, required O_2, and was inhibited by NADH, FAD and ATP. Addition of NADPH to the reaction mixture did not change measured activity. Specificity of nicotine synthase is unknown but is important because if MPO oxidizes both putrescine and N-methylputrescine then enzyme specificity dictates that the N-methyl-Δ'-pyrrolinium ion is utilized and

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nicotine is formed rather than nornicotine. Anabasine and anatabine are formed by similar decarboxylation condensation reactions and it is not known if these reactions are catalyzed by the same enzyme.

Cell and Tissue Culture Supplementation. Nicotine accumulates in callus and cell cultures of tobacco (Dawson, 1960; Speake et al., 1964; Krikorian and Steward, 1969, Neumann and Muller, 1971) as well as intact plants. Nicotine concentrations are influenced by the concentrations of growth regulators used for culture maintenance but generally are much lower than those found in plants (Furuya et al., 1967, 1971; Tabata et al., 1971; Takahashi and Yamada, 1973; Tabata and Hiraoka, 1976; Miller et al., 1983; Lockwood and Essa, 1984; Pinol et al., 1984). Where the relationship between auxin level, growth rate and nicotine concentration has been established, nicotine accumulation is in general inversely proportional to the growth rate of proliferating cells (Takahashi and Yamada, 1973).

Ravishankar and Malta (1982) obtained increased levels of nicotine (maximum level 0.1%) on both a culture and dry weight basis with increased urea concentration in the culture medium. The authors suggested that the increased nicotine levels may be the result of increased available ornithine for nicotine biosynthesis but this seems unlikely since added ornithine did not stimulate nicotine accumulation (Miller et al., 1983; Lockwood and Essa, 1984). Precursors for nicotine biosynthesis supplemented to culture media generally reduced nicotine accumulation (Miller et al., 1983). Even though callus from four isogenic burley tobacco genotypes differing in alkaloid content had much lower nicotine level than the plants from which the cells were derived, the nicotine level in the callus was proportional to the level in the respective plant. Based on the results from experiments of supplementing nicotine precursors into the culture medium without increasing alkaloid levels in any of the genotypes they concluded that control of nicotine accumulation in callus was not in a biosynthetic block in the pyridine or pyrrolidine ring formation. Lockwood and Essa (1984) also added nicotine precursors to the culture medium and measured decreased nicotine levels, but many precursors increased the level of anatabine. These authors reported the presence of

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myosmine, anabasine, anatabine anatalline and nicotelline in the cell suspension cultures.

Mantell et al. (1983) obtained 1 to 2% nicotine concentration in cell suspension cultures. They achieved these results by decreasing the growth regulator by 90%, increasing sucrose concentration to 50 g L^{-1} and reducing phosphate to 15 mg L^{-1} in the medium. They found that when sucrose was depleted from the medium nicotine accumulation stopped, but that when phosphate was depleted rapid nicotine accumulation occurred. Nicotine appeared to be synthesized in cells not undergoing cytokinesis and phosphate starved cells are arrested in the G_1 or to a lesser extent in G_2 stages of the cell cycle. These cells remained viable and apparently produced nicotine.

Leete et al. (1971) and Leete (1979) fed 5-fluoronicotinic acid to *N. tabacum* and found that 5-fluoronicotine and 5-fluoroanabasine were produced. Leete and Chedekel (1972) found that feeding N-methyl-Δ'- piperidine, which is not a normal precursor of anabasine, to *N. glauca* resulted in formation of N'-methylanabasine. Walton et al. (1988) reported that feeding cadaverine to hairy root cultures of *N. rustica* (*N. rustica* plants transformed with *Agrobacterium rhizogenes*) resulted in increased production of anabasine and inhibition of nicotine accumulation. We have obtained similar results by feeding cadaverine to intact plants of a low alkaloid *N. tabacum* genotype. From short-term diamine feeding of detached roots, demonstration of increased accumulation of alkaloid is more difficult. However, supplementing the feeding solution with nicotinic acid dramatically increased alkaloid accumulation. For example, when low alkaloid *N. tabacum* genotype roots were fed cadaverine alone for 7 hrs, anabasine concentration increased about 10 μg g^{-1} tissue dry wt, but with addition of 2 mM nicotinic acid to the cadaverine feeding solution anabasine increased to about 70 μg g^{-1}. Supplemental nicotinic acid alone increased anatabine levels. In *N. glauca* nicotine synthesis can be stimulated by feeding N-methylputrescine plus nicotinic acid (up to 8-times control level). Nicotine level increased only slightly with addition of only methylputrescine. Recently, Walton and McLauchlan (1990) reported that the diamine oxidase from *N. tabacum* hairy root cultures exhibited a high

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specificity for N-methylputrescine compared to cadaverine. In spite of this specificity, 1 mM cadaverine prevented the increased nicotine and nornicotine accumulation attributable to feeding 1 mM N-methylputrescine. They suggested that factors other than substrate specificity of MPO oxidase must be involved in the sensitivity of nicotine biosynthesis to perturbation. Our feeding studies with detached root cultures of *N. tabacum* and *N. glauca* fed N-methylputrescine and nicotinic acid indicate that MPO activity is not rate-limiting under these conditions and that penetration of exogenous diamine or availability of endogenous nicotinic acid is rate-limiting. Feeding of putrescine with or without nicotinic acid had little effect on nornicotine levels. Although putrescine can be oxidized to aminobutanal by tobacco root and leaf extracts containing MPO (Saunder and Bush, 1979), there is little, if any, evidence to support the idea that pyrroline is a normal precursor for nornicotine.

Alkaloid Transformations During Senescence and Curing

It should be noted that, in this review, senescence is defined to be initiated at the time when the plant is decapitated (induction of rapid nicotine biosynthesis and accumulation). The plant is still accumulating dry matter, but mainly in the upper leaves of the plant (Burton et al., 1983). Significant accumulation of the pyridine alkaloids is occurring in the upper leaves, but little or no alkaloid accumulation is occurring in the lower leaves. In addition to the accumulation of the four major pyridine alkaloids nicotine, anabasine, anatabine, and nornicotine, there is an increase in minor pyridine alkaloids (Burton et al., 1988; Andersen, et al. 1989). These include cotinine, 2,3'-bipridyl, N-acylnornicotines, N-acylanatabines, and N-acylanabasines (Figs. __.1, __.2). Even though there are only trace quantities of these minor alkaloids in mature leaves, the quantities increase significantly during air-curing (Burton et al., 1988).

Oxidized Pyridine Alkaloids. The two major oxidized alkaloids in tobacco are cotinine and 2,3'-bipyridyl. Cotinine is derived from nicotine by way of enzymatic or auto-oxidation (Enzell et al., 1977). Nicotine is conveniently converted to cotinine through auto-oxidation in low yield (Burton et al., 1987). The low level of cotinine

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in green tissue most likely is derived enzymatically rather than via auto-oxidation. Those tobaccos with relatively high content of nornicotine generally contain relatively high content of cotinine suggesting that the apparent P-450 enzymatic demethylation of nicotine to nornicotine may somehow be involved in cotinine formation as P-450 systems are required for cotinine formation in mammals. As plant maturity increases and during air-curing, there is greater accumulation of cotinine. There appears to be no effect of curing temperature on the accumulation of cotinine (Burton et al., 1988). 2,3'-Bipridyl is formed by oxidation of anatabine. It was first identified by Frankenburg et al. (1952) as a fermentation product along with 3-acetylpyridine. Even though 2,3'-bipyridyl accumulates during fermentation it is also present in low concentrations in green leaf (ca 2.0 ppm). Although there are detectable amounts of 2,3'-bipyridyl in mature leaf it is only approximately 0.001% of the level of anatabine. There is also a direct relationship between plant maturity and the level of 2,3'-bipyridyl (Burton et al., 1988). Curing environment influences the accumulation of this alkaloid. Curing tobacco at 32°C/83% relative humidity results in a 6-fold increase of 2,3'-bipyridyl in comparison to curing tobacco at 24°C/70% relative humidity. Curing tobacco at high relative humidity increases the moisture content of the cured lamina and enhance the fermentation process. These conditions result in the increased accumulation of 2,3'-bipyridyl. Other oxidized alkaloids present in green and cured tobacco include nicotyrine, 3-acetylpyridine, nicotinic acid, nicotinamide, and the nicotine-N-oxides. These oxidation products also increase during curing and have been reviewed (Enzell et al., 1977).

N-acylated Pyridine Alkaloids. A unique class of pyridine alkaloids that recently has been studied is the acylated derivatives of the secondary amine alkaloids - nornicotine, anatabine, and anabasine. The first acylated alkaloids identified in tobacco and shown in Figure 1 were n-hexanoyl- and n-octanoylnornicotine (Bolt, 1972). Warfield et al.(1972) identified N'-formyl- and N'-acetylnornicotine, Matsushima et al. (1983) isolated N'-butanoylnornicotine, Miyano et al. (1979) isolated N'-formylanatabine and N'-formylanabasine, and Matsushima et al. (1983) identified N'-(6-hydroxyoctanoyl)- and N'-(7-hydroxyoctanoyl)-nornicotine in cured tobaccos.

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The above acylated pyridine alkaloids are distributed within the leaf matrix. Severson et al. (1988) isolated and identified a series of C_{12} to C_{16} hydroxyacyl- and C_{12} and C_{13} acyl-nornicotines from the leaf surface of *N. repanda*, *N. stocktonii* and *N. nesophila*. The acylated compounds that contained a hydroxyl group at the 3 position of the acid side chain had greatest biological activity in insect, plant and microbial bioassays. The major component of the hydroxyacylnornicotines, [iso-N'-(3-hydroxy-12-methyltetradecanoyl)]-nornicotine is biosynthesized in the trichomes on the surface of the leaf (Zado and Jones, 1986) and not in the leaf matrix as the simpler acylated pyridine alkaloids. N'-formylnornicotine may be formed via formylation of nornicotine or oxidation of the N-methyl group of nicotine. This latter mechanism was proposed by Leete (1977) to explain conversion of nicotine to nornicotine. However, Burton et al. (1988) concluded that the accumulation profiles of N'-formylnornicotine, N'-acetylnornicotine and N'-formylanatabine were similar, suggesting that these compounds are formed via acylation of the secondary amine alkaloid.

Matsushima et al. (1983) first reported changes in levels of acylated nornicotine alkaloids. Except for 6-hydroxyoctanoyl- and 7-hydroxyoctanoylnornicotine, they reported decreased acylated nornicotines during curing. Burton et al. (1988) investigated the changes in the acylated alkaloids during senescence and air-curing of burley tobacco. In contrast to Matsushima's study, there was a significant increase of the acylated alkaloids throughout air-curing of burley tobacco. This observation was substantiated in a parallel study reported by Andersen et al. (1989).

Curing environment and plant maturity influence accumulation of these minor alkaloids. Tobacco harvested one week after decapitating (nicotine induction) contained 20% of the acylated alkaloids that was in the tobacco harvested four weeks after decapitation (Burton et al., 1988). Tobacco harvested seven weeks after decapitation did not contain significantly greater quantities of these alkaloids than tobacco from the 4-week induction treatment. Not only does leaf maturity affect accumulation of the acylated alkaloids, but curing environment also influences the accumulation of these alkaloids. Curing tobacco at elevated temperatures (32°C vs. 24°C) resulted in greater accumulation of the acylated alkaloids, regardless of plant

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maturity. Increases of these alkaloids generally occurred during the later stages of curing. This would indicate their accumulation occurred after the cell membranes lost integrity. Comparisons of acylated nornicotine data from air and flue cured tobacco also show curing environment influences the accumulation of these constituents (Burton et al., 1987). Flue curing tobacco resulted in reduced accumulation of these acylated derivatives. It should be noted that curing time interval for flue curing is approximately 7 days at above ambient temperatures, whereas air curing requires approximately 6 to 8 weeks for complete curing at ambient temperatures. The difference in curing environments would allow greater accumulation of acylated alkaloids to occur in the air curing environment.

The levels of acylated secondary alkaloids also vary in different tobacco genotypes. For example, the concentration of 6-and 7-hydroxyoctanoylnornicotines have been reported to accumulate in flue type tobacco in significant quantities (Djordjevic et al., 1990). In one cultivar the levels of these hydroxylated derivatives were the predominate acylated nornicotine. Burley tobacco cultivars contained detectable quantities of the hydroxylated derivatives but they were less than 1 ppm (unpublished data of authors). In general, the level of acylated alkaloids parallel the concentration of the secondary alkaloid content of the tobacco (Burton et al., 1988; Djordjevic et al., 1990).

In summary, the levels of the acylated secondary alkaloids are influenced by curing environment, plant maturity, genetics, and production practices. Even though the concentrations of these derivatives are small in comparison to the total alkaloid concentration in tobacco their accumulation may be of significance to flavor perception and reduction of the secondary amine alkaloids.

Tobacco-Specific Nitrosamines. Interest in tobacco-specific N'-nitrosamines (TSNA) has been a result of the report that some of these nitrosamines induced malignant tumors in mice, rats, and hamsters (Hecht et al., 1980). The nitrosamines of greatest interest have been N'-nitrosonornicotine (NNN), N'-nitrosoanatabine (NAT), N'-nitrosoanabasine (NAB), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Fig. __.9). It should be noted only NNN and NNK are reported to have

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significant tumorigenic activity. There have been many studies on the TSNA content and formation in tobacco yet conclusions from these studies in several instances are conflicting. NAT and NAB are formed directly from their precursor alkaloids anatabine and anabasine by reaction with nitrite. Concentrations of anabasine and anatabine are more than adequate for the amount of nitrosamine formed, since concentration of the alkaloids are at least 100-times greater than the reaction products. Also, the relative concentrations of NAT and NAB reflect the levels of anatabine and anabasine in the leaf (Djordjevic et al., 1989).

Proposed precursors and source of NNN have not been as straight forward. An initial study by Hecht et al. (1978) indicated nicotine as well as nornicotine were precursors of NNN. From radiolabeling they proposed nicotine was the major precursor of NNN. Caldwell et al. (1991) proposed nornicotine was the major precursor for NNN. This was based on data obtained from kinetic studies on the formation of NNN from nicotine and nornicotine. Studies by Djordjevic et al. (1989) and Andersen et al. (1989) showed the correlation coefficient between NNN and nornicotine was higher than between NNN and nicotine. Because of the complex nature of the tobacco matrix, additional studies on nitrosation of alkaloids in tobacco are needed.

NNK is derived from nicotine since it contains the methylamine group. It is formed by oxidation, ring opening between the C1' and C2' of the pyrrolidine ring, and nitrosation. The mechanism for this reaction has not been determined, therefore, it is not possible to discuss the key intermediates required for its formation. Treatment of nicotine and aqueous sodium nitrite with dilute hydrochloric acid (pH 5) will result in the accumulation of small quantities of NNK along with cotinine (Hecht et al., 1978; Burton, 1988). These observations show NNK does not require an enzymatic process for its formation.

Also, Hoffmann et al. (1980) reported that 46% of the NNN in tobacco was transferred to the smoke and the remaining pyrosynthesized. However, more recently Fischer et al. (1990) concluded that pyrosynthesis of NNN does not occur. No definitive experiment has been conducted to compare tobacco types for amount of

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TSNA accumulation. However, from various reports of the amounts of TSNA in tobacco, it is generally concluded that dark tobacco types have greatest TSNA concentration followed in descending order by burley, flue-cured and oriental tobacco (Fischer et al., 1989; Burton et al., 1990). These differences in TSNA accumulation probably do not reflect any genetic difference, but a culture difference of nitrogen fertilizer application and subsequent nitrate accumulation as a source of nitrite formation for nitrosation. There have been independent reports that the levels of TSNA in tobacco are directly related to alkaloids, nitrate, or nitrite content of the lamina. Djordjevic et al. (1989) and Andersen et al. (1989) found there was a direct relationship between alkaloids and TSNA in cured tobacco. Low levels of alkaloids in tobacco will result in reduced levels of TSNA, but production of low alkaloid tobacco is not practical since these tobaccos will not meet consumer acceptance. One would predict the positive relationship between alkaloids and TSNA in tobacco since pyridine alkaloids are direct precursors for TSNA.

Brunnemann et al. (1983) reported a positive and significant correlation between TSNA and nitrate in cigarette tobacco. In a nitrogen fertilization study, MacKown et al. (1984) reported no significant relationship between nitrate and TSNA even though there was a 10-fold difference in leaf nitrate. Fischer et al. (1989) reported there was a positive correlation between TSNA and nitrate from tobacco varieties and blended cigarette tobaccos. Therefore, nitrate level in tobaccos may be an indicator of TSNA accumulation. These observations suggest the reduction of nitrate to nitrite may be the rate-limiting step for TSNA accumulation.

Since nitrite is an immediate precursor of TSNA and since the alkaloids are present in 2 to 4 orders of magnitude higher concentration than nitrite, the obvious conclusion is that the major limiting factor for TSNA accumulation is nitrite. One could argue that nitrite is a transient reactive intermediate and there would be no correlation with TSNA accumulation, but this is not the case. In a study by Burton et al. (1989) on the curing of burley tobacco in a controlled environment of 32°C and 83% relative humidity, there was a significant increase of TSNA and nitrite in the plant tissue. In one instance the level of TSNA approached 1000 $\mu g\ g^{-1}$ and nitrite-N

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was 1.0 mg g^{-1}. This represented a 100-fold increase in TSNA and 1200-fold increase in nitrite over tobacco cured at ambient conditions. These temperatures and relative humidities certainly will support microbial activity in and on the tobacco matrix. It was assumed the nitrate reductase activity was from bacteria or fungi and not from residual nitrate reductase activity remaining in the plant tissue. Relationships between high levels of nitrite and TSNA have also been reported for smokeless tobaccos that were stored at elevated moisture content and 32°C (Andersen et al., 1989). This again supports nitrate reductase activity being responsible for nitrite accumulation.

The elevated TSNA and nitrite concentrations obtained from tobaccos stored at high moisture content are not representative of the levels obtained under normal air-curing conditions. Burton et al. (1990) conducted a 2-year study with 3 tobacco genotypes and several different tissue samples to determine the relationship between TSNA precursors and TSNA accumulation. They reported positive and significant correlations between the low endogenous levels of nitrite in tobacco and accumulation of individual TSNA. This positive correlation certainly is an over-simplification of the nitrosation reactions that occur in the tobacco matrix. For the reactions to occur the reactants must be in proximity to each other and most of the TSNA accumulation occurs after the cell membrane has become leaky. The average pH 6 of tobacco is not the ideal condition for optimum nitrosation and the moisture content is below optimum for the reaction to occur. However, even though optimum conditions do not prevail there is nitrosation of the tobacco alkaloids and accumulation of TSNA.

It was proposed that higher concentrations of TSNA in the leaf would be related to the concentration of the rate limiting precursor (Burton et al., 1992) and the distribution of these constituents within the leaf was determined. Leaves from an air cured dark tobacco was segmented into 4 cm x 7 cm segments and the midvein was portioned into 7 cm lengths. Surface plots were calculated from the analytical data obtained for these constituents in each leaf segment. Alkaloid levels were greatest on the periphery of the leaf and lowest along the midvein. Nornicotine is shown as a representative accumulation pattern of each of the alkaloids (Fig. __.10A). TSNA levels were lowest at the tip of the leaf and highest in the midvein at the base of the

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leaf (Fig. __.10B). Nitrate concentration was greatest along the midvein. Nitrite concentrations were highest in midvein at the base of the leaf and more closely paralleled the profile of TSNA. When the TSNA and nitrite data are presented as percentage distribution in the leaf, the patterns are almost identical (Figs. __.10C, __.10D). Both TSNA and nitrite are greatest in the midvein at the base of the leaf. These observations suggest that the nitrosation of alkaloids in tobacco leaf is dependent on the capacity for nitrite production within the system. Alkaloids and nitrate are essential for nitrosamine formation, but if nitrite is not produced, no nitrosamines will be formed.

This explanation for nitrosation of the alkaloids is somewhat speculative at this time. We are proposing that in stalk-cut air-cured tobacco the midvein attached to the stalk is the last part of the leaf to dry. Maintaining moisture over a prolonged time may allow microbial activity to occur. Some of these microbes reduce nitrate (Parsons et al., 1986) and could explain the increased accumulation of nitrite at the base of the leaf. Preliminary results from primed air-cured segmented leaf which lose moisture more uniformly support this speculation. The conditions required for the accumulation of TSNA have not been totally clarified but it is apparent that disruption of plant cell membrane and apparent microbial reduction of nitrate to nitrite is required for nitrosation of the alkaloids to occur. From present knowledge, it will be possible to produce tobacco with predictable levels of TSNA.

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Captions for Illustrations

Figure __.1. Structures of selected *Nicotiana* alkaloids.

Figure __.2. Derivatives of nornicotine, anabasine and anatabine.

Figure __.3. Nicotinic acid from aspartic acid and a three carbon glycerol derivative.

Figure __.4. Synthesis of N-methyl-Δ'-pyrrolinium ion.

Figure __.5. Formation of (-)-2'S-nicotine, nornicotine and myosmine from nicotinic acid and N-methyl-Δ'-pyrrolinium ion.

Figure __.6. Anatabine formation from nicotinic acid.

Figure __.7. Involvement of putrescine in metabolic pathways of *Nicotiana spp.*

Figure __.8. Pyridine nucleotide cycle and nicotinic acid availability for nicotine biosynthesis.

Figure __.9. Major N'-nitrosamines derived from *Nicotiana* alkaloids.

Figure __.10. Distribution of (A) nornicotine, (B) tobacco-specific nitrosamines and percentage distribution of (C) nitrite, and (D) tobacco-specific nitrosamines in cured leaf. X axis coordinates 0, 0 are the tip of the leaf with midrib samples on the line from coordinates 0, 0 to 0, 70.

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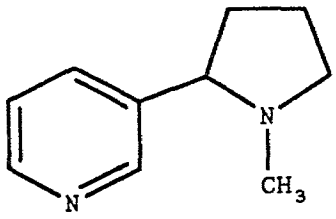
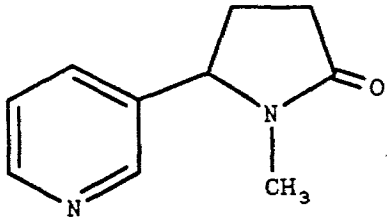
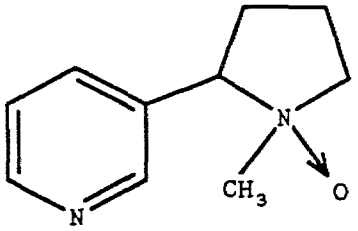
Figure 1



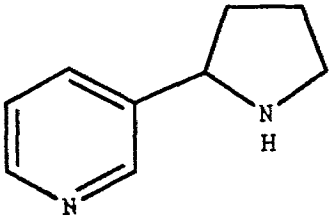
Nicotine



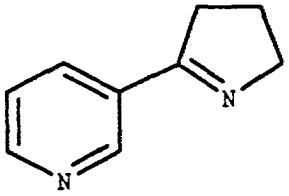
Cotinine



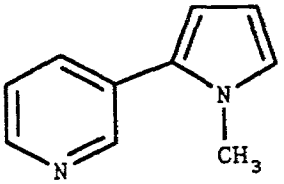
Nicotine N'-oxide



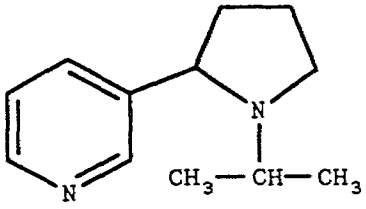
Nornicotine



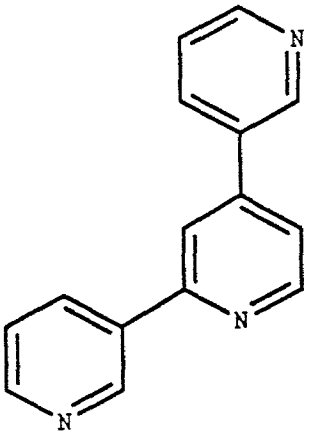
Myosmine



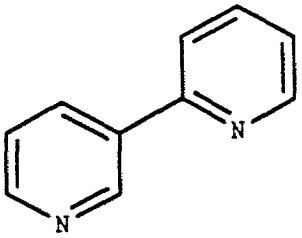
Nicotyrine



N'-isopropylnornicotine



Nicotelline



2,3'-bipyridyl

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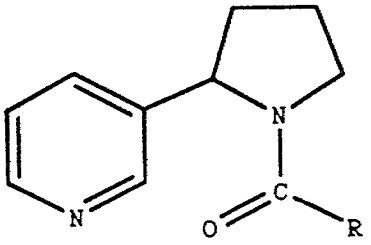
Figure 2



N'-acylnornicotines

R

-H

$-CH_3$

$-CH_2CH_3$

$-(CH_2)_2CH_3$

$-(CH_2)_4CH_3$

$-(CH_2)_6CH_3$

$-(CH_2)_4CH(OH)CH_2CH_3$

$-(CH_2)_5CH(OH)CH_3$

$-CH_2-CH(OH)-(CH_2)_8CH_3$

$-CH_2-CH(OH)-(CH_2)_9CH_3$

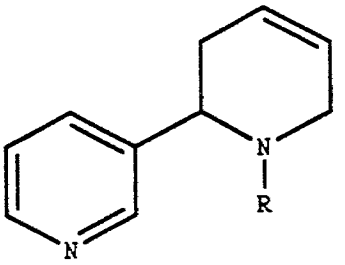
$-CH_2-CH(OH)-(CH_2)_{10}CH_3$

$-CH_2-CH(OH)-(CH_2)_{11}CH_3$

$-CH_2-CH(OH)-(CH_2)_{12}CH_3$

$-(CH_2)_{10}CH_3$

$-(CH_2)_{11}CH_3$

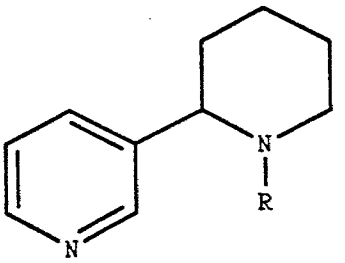


R

H, Anatabine

CH_3, Methylanatabine

COH, N'-formylanatabine



R

H, Anabasine

CH_3, Methylanabasine

COH, N'-formylanabasine

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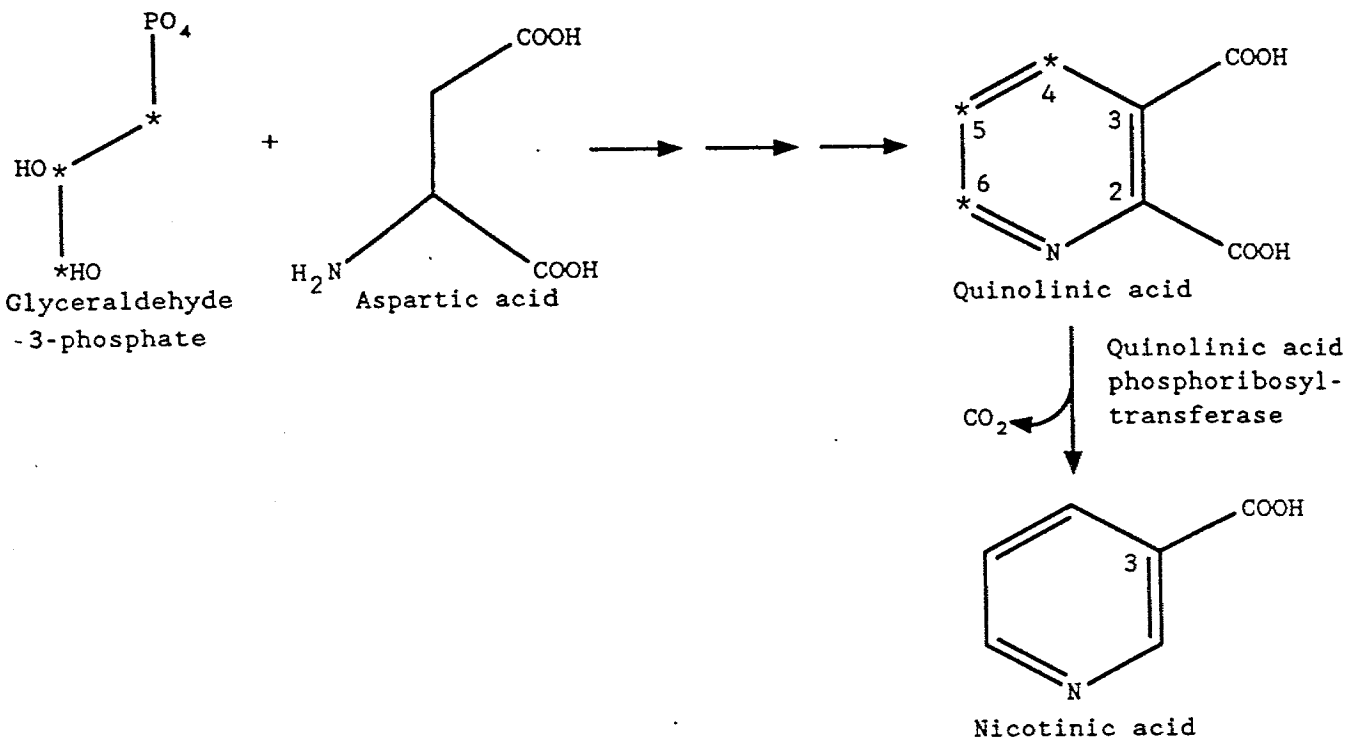
Figure 3



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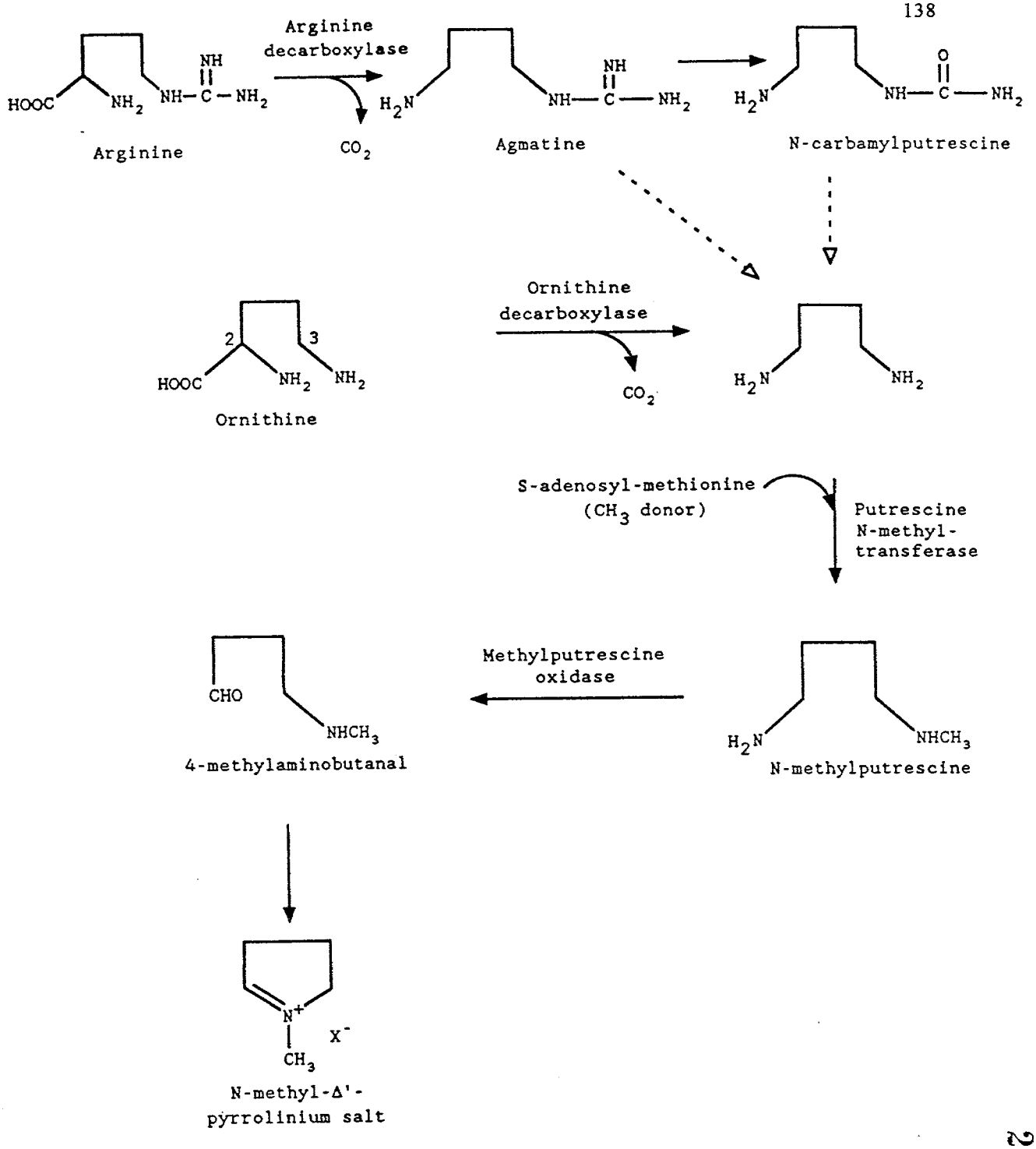

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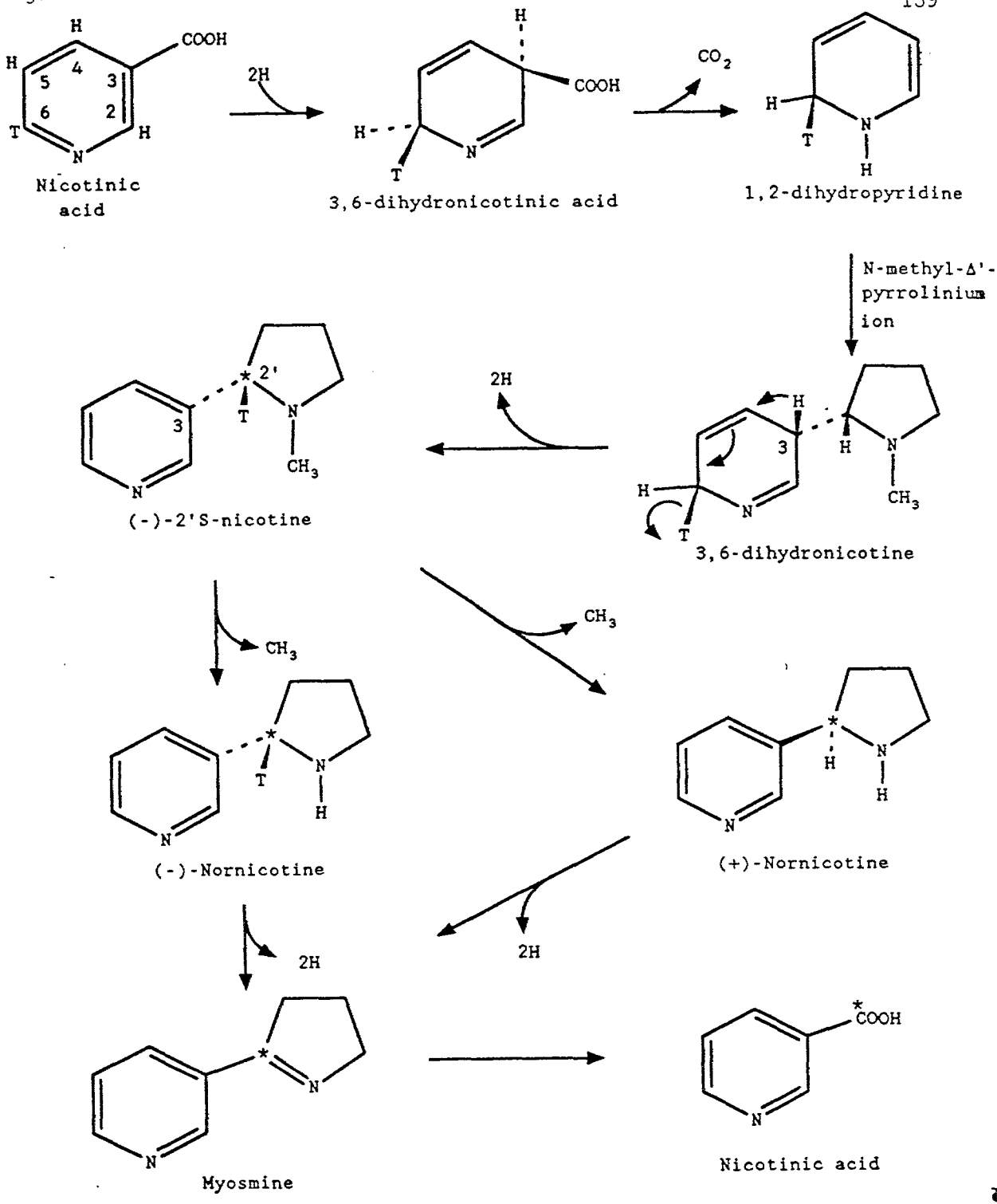

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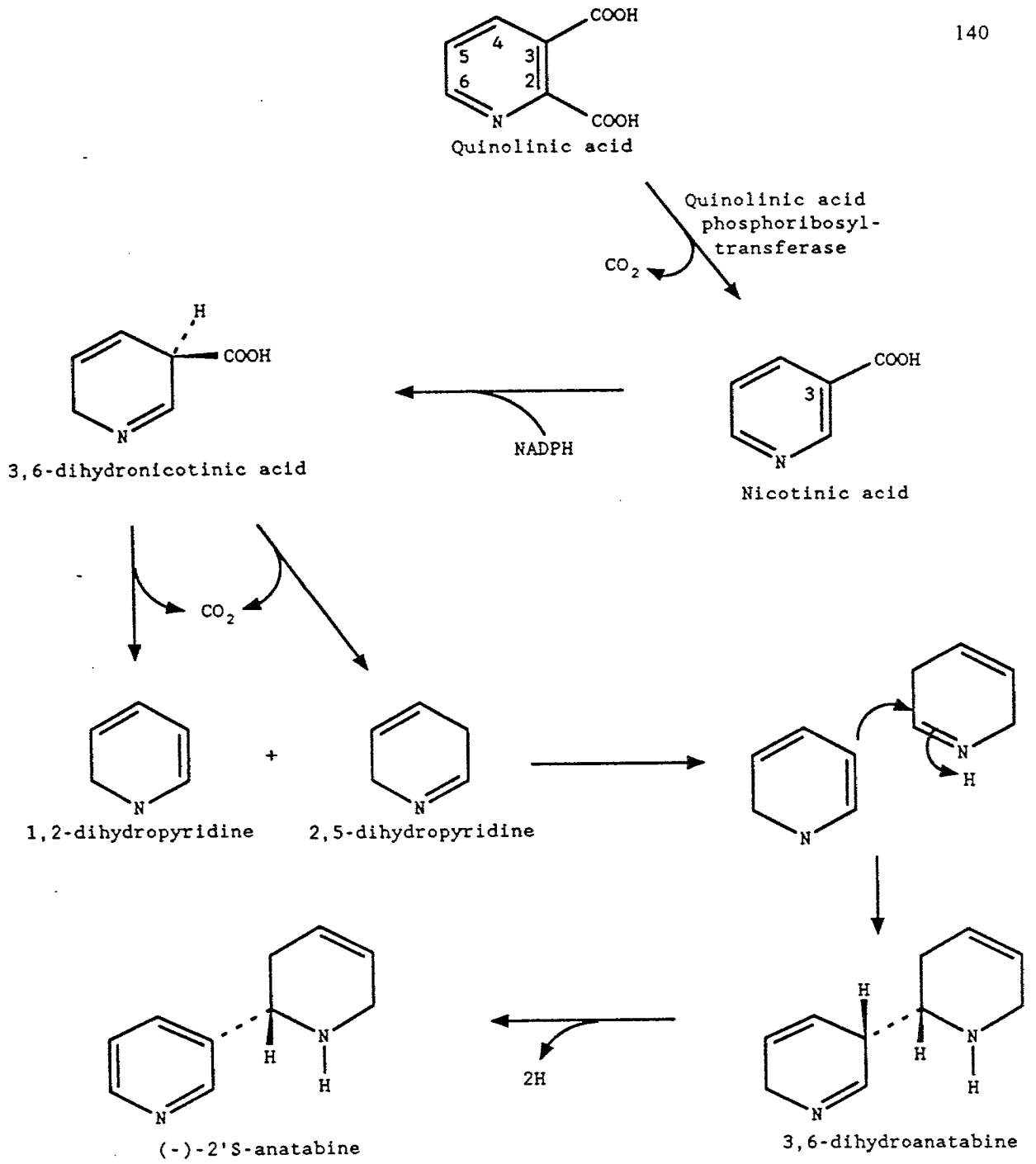

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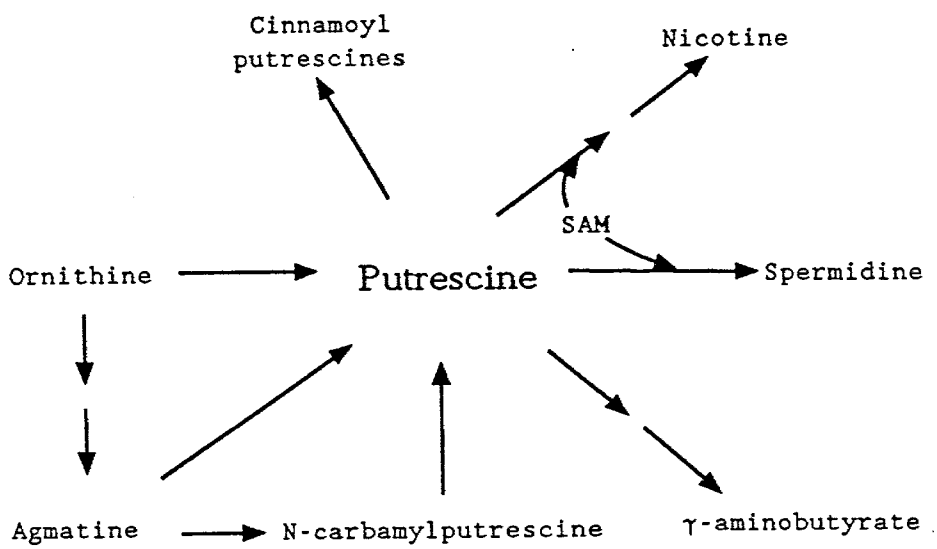

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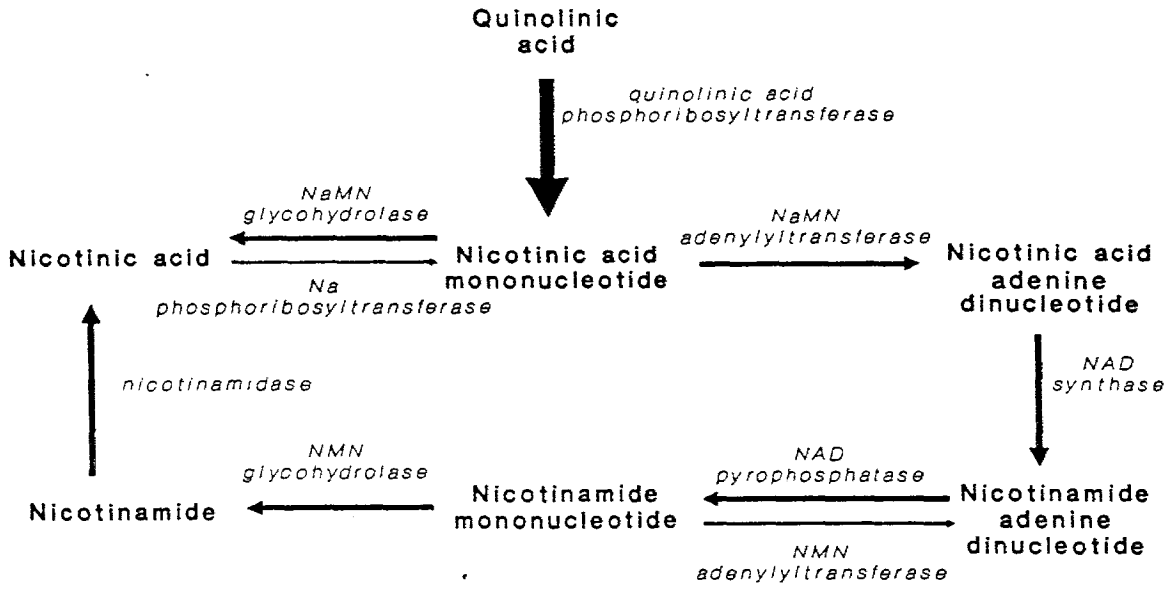

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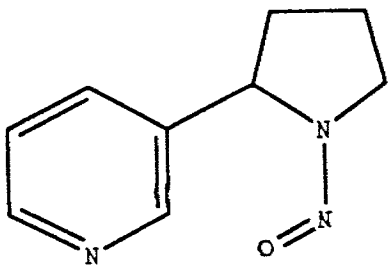

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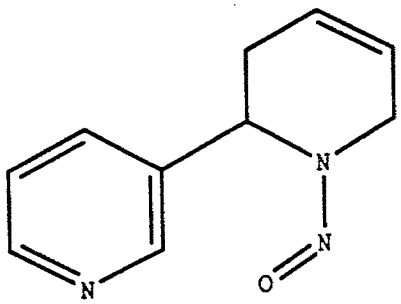

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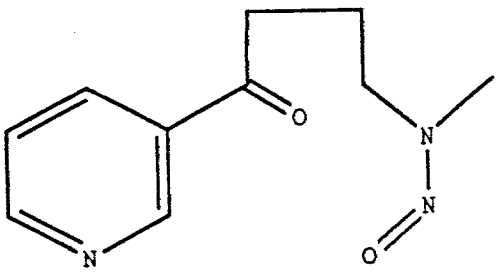

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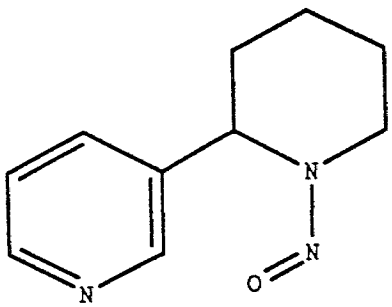

NAB

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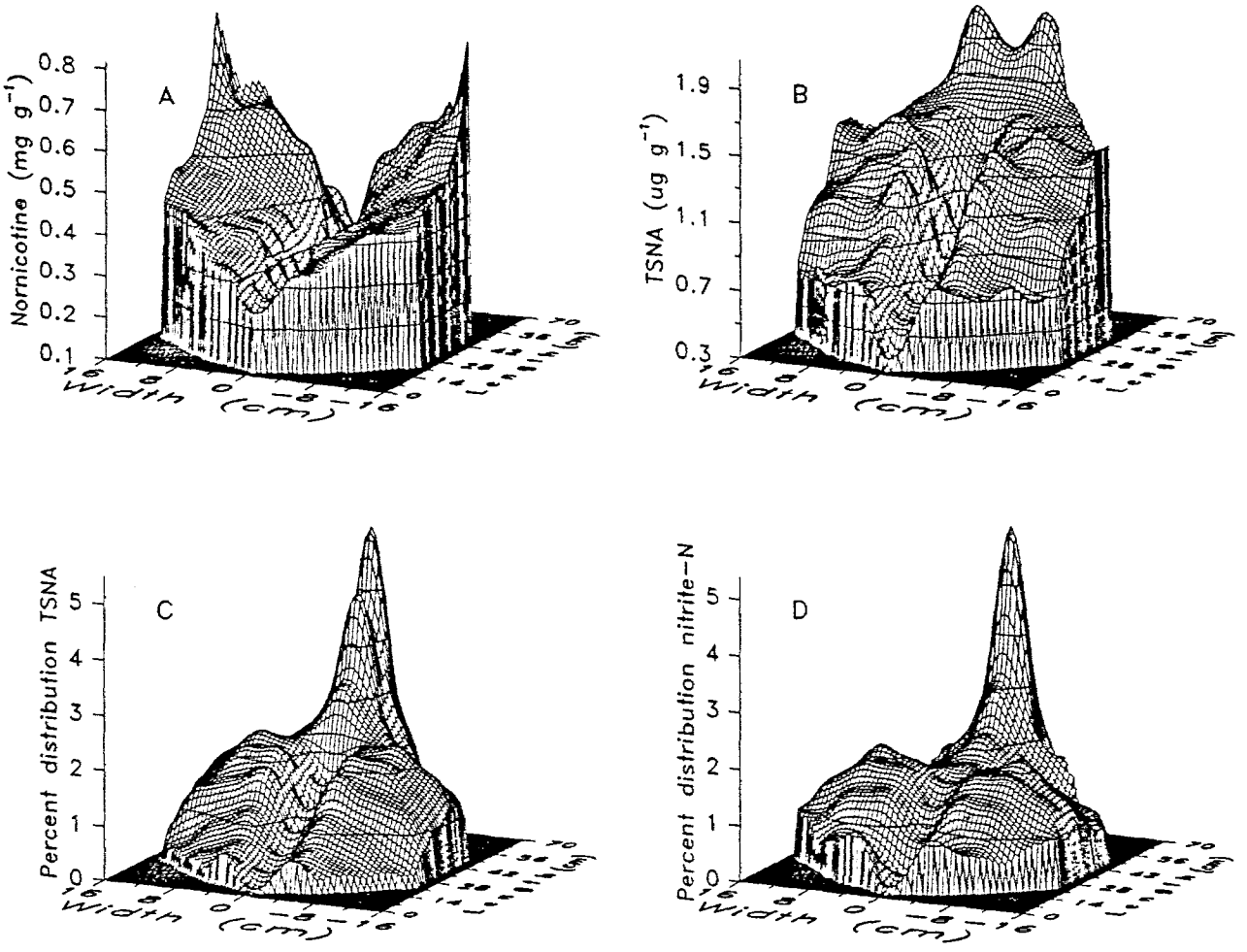

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